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The Role of a Surface Glycoprotein in the Interaction of Aggregatibacter actinomycetemcomitans with Dendritic Cells

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THE ROLE OF A SURFACE GLYCOPROTEIN IN THE INTERACTION OF *AGGREGATIBACTER ACTINOMYCETEMCOMITANS* WITH DENDRITIC CELLS

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ABSTRACT

Periodontal diseases are a group of prevalent inflammatory diseases affecting the tissues supporting and surrounding the teeth. In periodontal diseases, the inflammatory response of the periodontal tissues is induced by a polymicrobial biofilm formed at or below the gingival margin. *Aggregatibacter actinomycetemcomitans*, a Gram-negative oral pathogen identified within this biofilm, is a causative agent of both chronic and localized aggressive periodontitis. Dissemination of *A. actinomycetemcomitans* from the oral cavity also initiates multiple systemic infections, including soft tissues abscesses, pneumonia and endocarditis.

The innate immune response is the first line of defense against bacterial infections. Dendritic cells, a group of professional antigen-presenting cells of the innate immune system, express multiple surface C-type lectin receptors for the recognition of glycoproteins associated with bacterial cells. Internalization of the organism by receptor-mediated endocytosis results in phagolysosome formation and degradation of the bacteria for antigen presentation. Surface displayed antigens presented in an MHC complex to antigen-specific T lymphocytes initiate an adaptive immune response.

A. actinomycetemcomitans expresses surface structures Extracellular matrix protein adhesin A (EmaA), which extends from the cell envelope, and is composed of three identical glycoproteins. The role of these structures in the interaction of *A. actinomycetemcomitans* with dendritic cells is understudied. A series of experiments presented here investigated the interaction of *A. actinomycetemcomitans* with dendritic cells and the role of EmaA in this process. *A. actinomycetemcomitans* was internalized and found to survive within dendritic cells. The internalization of bacteria was observed to be associated with the presence of EmaA. Fewer *emaA* mutant bacteria were recovered from dendritic cells when compared with the parent strain. *In trans* complementation of the *emaA* mutant strain restored the interactive and survival capability of the bacteria. These data suggest that EmaA mediates the interaction of *A. actinomycetemcomitans* with dendritic cells. The migration of dendritic cells to draining lymph nodes suggests that the EmaA-mediated internalization and intracellular survival is a potential mechanism for the immune evasion and *in vivo* dissemination of *A. actinomycetemcomitans.*

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CHAPTER 1: INTRODUCTION

1.1. Oral health and diseases

Oral health is important for many physiological tasks, including the initial processing of food, being able to speak clearly and fluently during communication, and the expression of feelings [1]. Moreover, there is a strong linkage between oral health and the overall health of the body [2]. The oral cavity is suggested to serve as a reservoir for oral pathogens to disseminate and cause multiple systemic diseases [2]. Over 100 systemic diseases, including cardiovascular disease, diabetes and nutritional problems, have oral manifestations [2].

The tooth is divided into two regions, the crown and the root. Both sections are formed by dentin with a chamber inside containing dental pulp. The crown, the upper region of the tooth, is composed of dentin and covered with enamel, a hard material mainly composed of hydroxyapatite. The lower region comprising the root is covered with cementum, which is composed of inorganic material (hydroxyapatite) and organic portion (collagen and proteoglycans) [3]. In the mouth, the teeth are embedded in the jaw and supported by the alveolar bone. Periodontal ligaments, a connective tissue mainly composed of bundled collagen fibers, integrate the tooth root and the alveolar bone [3]. The gingiva is the soft tissue surrounding the tooth that acts as a seal for the root and the alveolar bone. The shallow space between the gingiva and the tooth is called the gingival crevice. Crevicular fluid, an inflammatory exudate in the gingival crevice with serum-like components, serves as a mechanism to defend the tissues against oral pathogens [4]. Inflammatory molecules, such as cytokines and chemokines in the crevicular fluid can be analyzed to assess the extent of periodontal diseases [4].

The oral cavity is a habitat that supports the growth of over seven hundred taxa of microbes, including bacteria, fungi, protozoa and viruses [5]. Within minutes after we clean the teeth, the proteins in the saliva coat the teeth and form a pellicle. This proteinaceous film formed on the teeth surface provides a substrate for the formation of a dental biofilm by oral microbes [6]. The composition of dental biofilms consists of oral commensals and are typically non-pathogenic. Regular oral hygiene practices, including teeth brushing, interdental flossing and regular check-ups prevent the build-up of biofilms. The host immune system and the commensals in the biofilm also help to limit the growth of oral pathogens and maintain oral health [6]. However, in situations such as poor oral hygiene or immune deficiencies, an imbalance in the oral biofilm can initiate an overgrowth of pathogens and results in multiple oral diseases, with the two major forms being dental caries and periodontal diseases [6].

Dental caries are caused by specific Gram-positive bacteria including *Streptococcus mutans*, *Streptococcus sorbrinus* and *Lactobacillus casei* [7]. These bacteria are able to metabolize the fermentable carbohydrates in food debris, including sucrose, fructose and glucose, to lactic acid [8]. Due to the low pH of the lactic acid, the enamel and dentin will be dissolved, causing the demineralization of these materials and the formation of dental caries [8]. Other oral pathogens can infect the tissues surrounding the tooth, leading to periodontal diseases.

1.1.1. Periodontal diseases

Periodontal diseases are a group of inflammatory diseases affecting the tissues surrounding and supporting the teeth including the cementum, periodontal ligament, alveolar bone and gingiva [9]. Periodontal diseases are present in more than two thirds

of the population in the US [10]. All people above the age of 40 have some form of periodontal diseases [10]. Risk factors for periodontal diseases include poor oral hygiene, smoking, age and immunocompromised conditions [11, 12]. An early form of periodontal diseases is gingivitis. Gingivitis is caused by the formation of dental biofilms and subsequent immune response to the biofilms [13]. The symptoms of gingivitis include redness, swelling, pain and bleeding of the gum [13]. Bad breath, or halitosis, is also a sign of gingivitis [13]. There is no destruction of periodontal tissues in gingivitis and the symptoms are reversible if treated. However, without proper treatment, gingivitis may progress to a more severe form of periodontal diseases - periodontitis.

1.1.2. Periodontitis

In contrast to gingivitis, periodontitis is associated with irreversible destruction of the periodontal tissues, including loss of the alveolar bone and periodontal ligaments [14]. This tissue destruction is caused by the immune response to specific species of bacteria in the dental biofilm [14]. The inflammatory responses and secretion of pro-inflammatory cytokines induce the proliferation of osteoclast cells and inhibit the proliferation of osteoblast cells, which together lead to the destruction of the alveolar bone [15]. With the progressive destruction of the periodontal tissues, the gingival sulcus becomes deeper (from 1-2 mm to 3-7 mm) and forms a periodontal pocket. With the progression of tissue destruction, the teeth lose the support of the periodontium and eventually fall out of the jaw.

1.2. Oral biofilm and periodontal pathogens

Biofilms are surface-attached or self-attached microbial communities embedded in a matrix called extracellular polymeric substance (EPS) [16]. Biofilms are

advantageous for the microbes within the biofilm as it enhances microbial tolerance to antibiotic agents, physical removal and attack by the immune system [17]. Formation of the biofilm is initiated when a single planktonic bacterium samples the environment with weak, non-specific interactions, such as van der Waals forces and charge-charge interactions (Figure 1.1) [22]. If the environment is suitable, the bacterium adheres to the substrate using surface proteins or structures including fimbriae (pili) and non-fimbrial adhesins, which mediate much stronger interactions [19]. These bacteria (first colonizers) will grow and replicate, forming a microcolony. At this stage, those bacteria that cannot adhere to abiotic surfaces but are able to bind to other bacteria, termed secondary colonizers, will co-aggregate and add to the biomass [22]. During growth of the microcolonies, bacteria in the biofilm will secrete proteins, polysaccharides and DNA, which together form the EPS that can also mix with the components from the environment [17, 33].

EPS holds the microcolony together to protect the biofilm from the environment and provides a relatively isolated environment within the biofilm. Bacteria in the biofilm produce signaling molecules called autoinducers for communication with each other. The signaling pathways or quorum sensing take place in the biofilm to coordinate gene expression of the individual bacteria within the community. Therefore, the gene expression profile of the biofilm-associated bacteria can be dramatically different from bacteria grown in a planktonic fashion [18]. Horizontal gene transfer also occurs more frequently within biofilms, allowing microbes within the biofilm to gain new potential functions more rapidly and enhancing the stability of the biofilm [20].

When bacteria in the biofilm die and the cells lyse, DNA is released and integrated into the EPS [22]. The microcolonies will continue to grow and form a three dimensional mushroom-like structure, with channels in the base to allow for flow of nutrients throughout the biofilm, thereby supporting bacterial growth. Microbes in the biofilm can also disseminate and become planktonic, allowing for the disposal of the biofilm to distant sites [21].

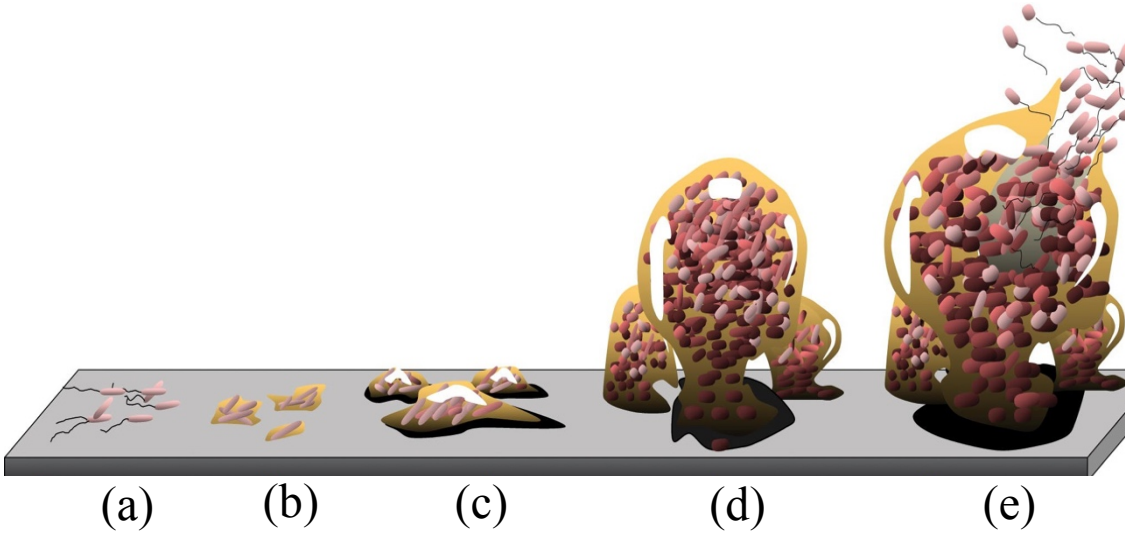

Figure 1.1. Development of a biofilm. Formation of a biofilm is initiated when planktonic bacteria sample the substrate and adhere to the substrate using adhesins (a). The attached bacteria grow and form microcolonies together with the incoming bacteria (b). The microbes in the biofilm secrete proteins and polysaccharides, which form a matrix covering the biofilm and protecting it from the environment (c). The microcolonies grow and form a three-dimensional structure with channels on the bottom, allowing for the flow of nutrients (d). At the late stage of biofilm formation, microbes can leave the biofilm and become planktonic again (e). Image adapted from Monroe, D., *PLoS Biol*, 2007, 5(11):2458-2461

The surface of teeth and the salivary proteins coating teeth provide a hospitable substrate for biofilm formation [22]. Gram-positive bacteria, usually oral commensals such as *Streptococcus parasanguinis* and *Streptococcus gondonii*, act as early colonizers in the biofilm formation and adhere to the surface of the teeth by fimbriae [22]. Following

maturation of the biofilm, the Gram-negative bacterium *Fusobacterium nucleatum* co-aggregates with the early colonizers, acting as an anchor and bridge for the interaction of multiple late colonizers onto the biofilm [22, 25]. With the formation of the biofilm, bacterial metabolism changes the micro-environment, decreases the oxygen concentration in the environment, and facilitates the growth of Gram-negative anaerobic bacteria [22]. These Gram-negative bacteria include *Porphyromonas gingivalis*, *Treponema dendicola*, *Tennerella forsythia*, and *Aggregatibacter actinomycetemcomitans*, which are associated with periodontitis [14].

1.3. *Aggregatibacter actinomycetemcomitans*

A. actinomycetemcomitans is a fastidious, capnophilic, non-motile coccobacillus (bacterium with a morphology intermediate between cocci and bacilli). This bacterium is a late colonizer in the formation of the dental biofilm. The average size of an *A. actinomycetemcomitans* bacterium is 1.0 μm by 0.5 μm, as determined by electron microscopy (Figure 1.2) [24]. In addition to chronic periodontitis, *A. actinomycetemcomitans* is closely associated with an acute form of periodontal disease, localized aggressive periodontitis (LAP). LAP mainly affects individuals younger than 30 years old, with those of African descent being more susceptible [26]. The infection of LAP is limited to specific teeth, i.e. incisors and first molars. LAP caused by *A. actinomycetemcomitans* is able to lead to the loss of teeth in a few months if left untreated, dramatically faster than the progression of chronic periodontal diseases, which may take decades [26].

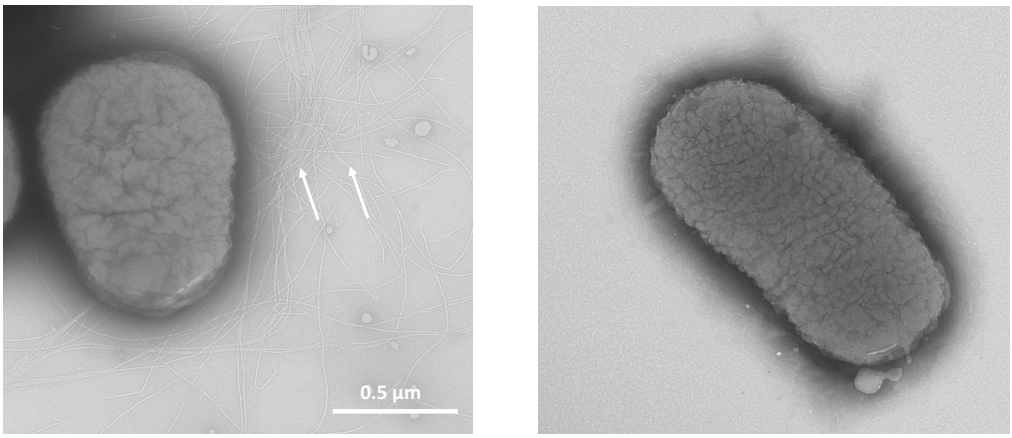

Figure 1.2. Fimbriated and afimbriated strains of *A. actinomycetemcomitans.* Electron microscopy images of fimbriated (left) and afimbriated (right) *A. actinomycetemcomitans* are presented. White arrows point to the bundled fimbriae, which are involved in biofilm formation and tissue adhesion. Images courtesy of Dr. Teresa Ruiz (University of Vermont).

A. actinomycetemcomitans is able to disseminate from the oral cavity and enter the circulation to cause multiple extra-oral infections including soft tissue abscesses, pneumonia, bone inflammation and infective endocarditis [27]. The infections caused by *A. actinomycetemcomitans* are attributed to multiple virulence factors, molecules that are produced by the bacteria and contribute to the pathogenesis. These virulence factors include protein toxins, lipopolysaccharide, and adhesins [27].

1.3.1. Virulence factors of *Aggregatibacter actinomycetemcomitans*

1.3.1a Toxins

Leukotoxin (LtxA) is a ~113 kDa secreted protein that is specific for leukocytes [28]. It has ~50% sequence homology to the alpha-hemolysin of *E. coli* and is a member of the repeats-in-toxin (RTX) family [29]. The name of the toxin family is based on the glycine-rich repeats in the amino acid sequences. These glycine repeats bind calcium ions, which are required for the activity of the toxin [29]. The biosynthesis and secretion of the leukotoxin is mediated by the *ltxCABD* operon, which encodes the toxin molecule

(LtxA) and two components of a Type I secretion system (LtxB and LtxD) [30]. In the *ltx* operon, the synthesized LtxA is modified by acylation on the two internal lysine residues by LtxC. This acylation is essential for the activity of the LtxA molecule [31].

The secretion apparatus of leukotoxin is composed of three proteins: LtxB, LtxD and TolC (TdeA). LtxB is an inner membrane ATPase and provides energy for the secretion of LtxA [32]. Upon interaction with LtxA, LtxB undergoes conformational changes and interacts with the periplasmic protein LtxD to form a channel in the periplasm to allow for the transport of LtxA through the periplasm. The LtxA is finally transported through a trimeric outer membrane pore formed by TdeA, a homolog of TolC [32].

The production of leukotoxin is strain dependent: JP2 strains of *A. actinomycetemcomitans* have a high production of leukotoxin, whereas strain 652 has minimal leukotoxin production [34]. The major difference between the two groups is that JP2 strains have a 530 bp deletion in the 3' region of the *ltx* promoter [34]. The detailed mechanism for the control of leukotoxin production at the genetic level is still under investigation. An inner membrane protein MorC was found to be involved in the secretion of leukotoxin; mutation of *morC* led to a significant decrease of LtxA production without impacting the mRNA level of *ltxA* [53]. Interestingly, leukotoxin can also be present in the outer membrane vesicles, which represent another delivery route for this toxin [33]. The leukotoxin induces the apoptosis of the leukocytes, thus contributing to the immune evasion of *A. actinomycetemcomitans* [29].

Cytolethal distending toxin (CDT) is an A-B toxin that is able to degrade DNA of the host cells [35]. The effects of CDT on the host cells include stall of the cell cycle,

nuclear swelling, chromatin fragmentation, and eventually apoptosis of the host cell [35]. CDT is encoded on a three-gene locus *cdtABC* and the active toxin subunit is CdtB, which has DNase I activity [38]. Although the roles of CdtA and CdtC are still unknown, these proteins are required for maximal toxic activity [35]. Leukocytes are susceptible to CDT, suggesting that CDT also contributes to the immune evasion of *A. actinomycetemcomitans*.

1.3.1b Lipopolysaccharide

Lipopolysaccharide (LPS) is the major component of the outer membrane of Gram-negative bacteria, covering 75% of the bacterial surface and contributes to the maintenance of membrane stability [39]. An LPS molecule is composed of lipid A, core oligosaccharide and O-polysaccharide (O-PS). LPS molecules make up the outer leaflet of the outer membrane together with outer membrane proteins [39]. Lipid A is composed of a disaccharide decorated with fatty acids and it anchors the LPS molecule into the bacterial membrane [39]. The core oligosaccharide links the O-PS to Lipid A. The O-PS, or O-antigen, is composed of repeated glycan oligomers and is a major antigen for antibody recognition [35]. The composition of the O-PS allows for the segregation of *A. actinomycetemcomitans* into seven serotypes (a-g), with each serotype encoding an O-PS with unique composition and structure [41, 42]. Serotype b and c are the most prevalent strains associated with diseases caused by *A. actinomycetemcomitans* [41]. Dendritic cells co-cultured with serotype b strains expressed higher level of cytokines, including IFNγ, TNFα, IL-1β, IL-12, IL-6, and IL-23 compared to the cells co-culture with equal amount of serotype a and serotype c strains [43]. The results held true for dendritic cells co-cultured with purified LPS from the three serotypes individually,

suggesting that the different cytokine expression levels are attributed to the LPS of the different serotypes [43].

1.3.1c Non-fimbrial adhesins

A. actinomycetemcomitans produces three known non-fimbrial adhesins: Aae, ApiA and EmaA. All of these adhesins are autotransporter proteins, which have all the information encoded on the amino acid sequence for outer membrane translocation and secretion of the protein [36]. An autotransporter protein is composed of an N-terminal signal sequence, a passenger domain and a C-terminal membrane anchor domain [36]. Autotransporters are divided into three classes: Type Va, Type Vb and Type Vc according to different mechanisms for secretion [36]. Type Va, or conventional autotransporter protein, encodes a membrane anchor domain containing 14 β-strands. This domain is able to form a β-barrel in the outer membrane to allow for the translocation of the passenger domain. The adhesin Aae is a 90 kDa monomeric Type Va autotransporter. Type Vb autotransporters have separate genes encoding the passenger domain and membrane anchor domain [36]. Both domains have signal sequences for the inner membrane targeting and translocation. After translocating into the periplasm, the two domains interact with each other and initiate membrane translocation. Type Vc proteins, also called trimeric autotranporters, contain four β-strands in the translocator domain that are required to trimerize to form a complete β-barrel, so the mature molecules are homotrimers [36]. The other two characterized non-fimbrial adhesins in *A. actinomycetemcomitans* are the 34 kDa ApiA and the 202 kDa EmaA, which are both Type Vc autotransporters [44].

Both Aae and ApiA mediate the adhesion of *A. actinomycetemcomitans* to buccal epithelial cells. Double knockout of *aae* and *apiA* completely abolished the epithelium adhesion of this bacterium [37]. Additionally, ApiA also contributes to the resistance of *A. actinomycetemcomitans* to the complement system [44]. EmaA is an adhesin that mediates binding of *A. actinomycetemcomitans* to the extracellular matrix (ECM). The detailed characteristics of EmaA will be described in Chapter 1.5.

1.4. Fimbriae of *Aggregatibacter actinomycetemcomitans*

Clinical isolates of *A. actinomycetemcomitans* collected from periodontal pockets are characterized by a rough colony surface, irregular edge and a star shaped internal structures in the center of the colony [24, 45]. The colony is not easily disrupted by physical removal with an inoculating loop. When grown in liquid culture, these clinical strains grow on the surface of the container instead of forming a suspension in the liquid phase, leaving the culture supernatant essentially clear (Figure 1.3). These distinguishing phenotypes are attributed to the production of long bundled structures termed fimbriae [45, 46]. The fimbriae produced by *A. actinomycetemcomitans* are 5-7 nm in diameter and can be several micrometers in length (in comparison with 1 μm, which is the average size of an *A. actinomycetemcomitans* cell) [46]. The fimbriae are involved in the auto-aggregation, adhesion to substrates and biofilm formation [46].

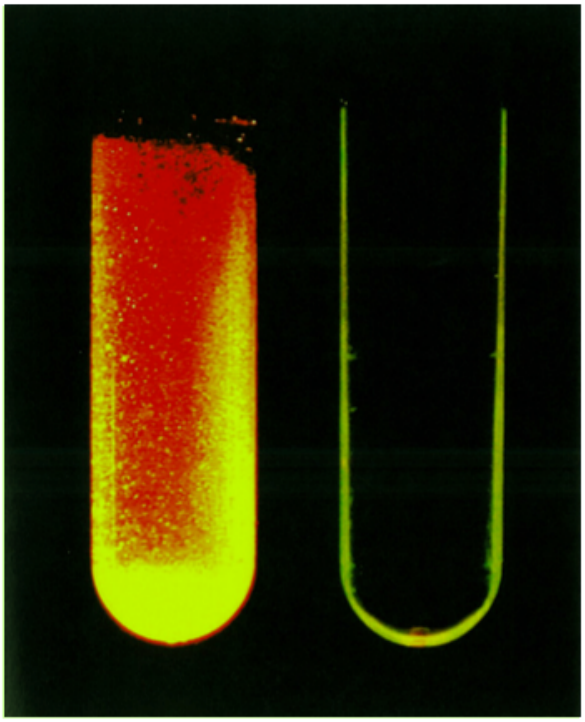

Figure 1.3. Biofilm formed by fimbriated *A. actinomycetemcomitans.* Fimbriated (left) and afimbriated (right) *A. actinomycetemcomitans* were grown in liquid media overnight and the supernatant was removed. The biofilms formed on the surface of the tubes were stained with ethidium bromide and visualized by UV. Image adapted from Figurski D. H. et al. *INTECH Open Access Publisher*, 2013.

1.4.1 The biosynthesis and secretion of fimbriae in *A. actinomycetemcomitans*

The fimbria of *A. actinomycetemcomitans* is a member of the type IV pili family and is synthesized and secreted by a 14 gene locus called the Tight adherence (Tad) locus (Figure 1.4) [49]. The secretion apparatus is homologous to the type II secretion system, which is a common secretion system used by multiple Gram-negative bacteria including *P. aeruginosa* and *Klebsiella pneumoniae* for the secretion of virulence factors such as proteases and lipases [50, 51]. The first gene in the locus, *flp-1*, encodes a 6.5 kDa protein Flp1, which is the subunit of the fimbriae [52]. The Flp1 subunit protein has a long leader sequence, a hydrophobic domain involved in polymerization of the subunit and a C-

terminal functional domain involved in the adhesion activity [52]. The subsequent gene *flp-2* is a pseudogene and is the only dispensable gene in this locus [53]. The following gene, *tadV*, encodes a prepilin peptidase that cleaves the leader peptide of Flp1 during its maturation [54].

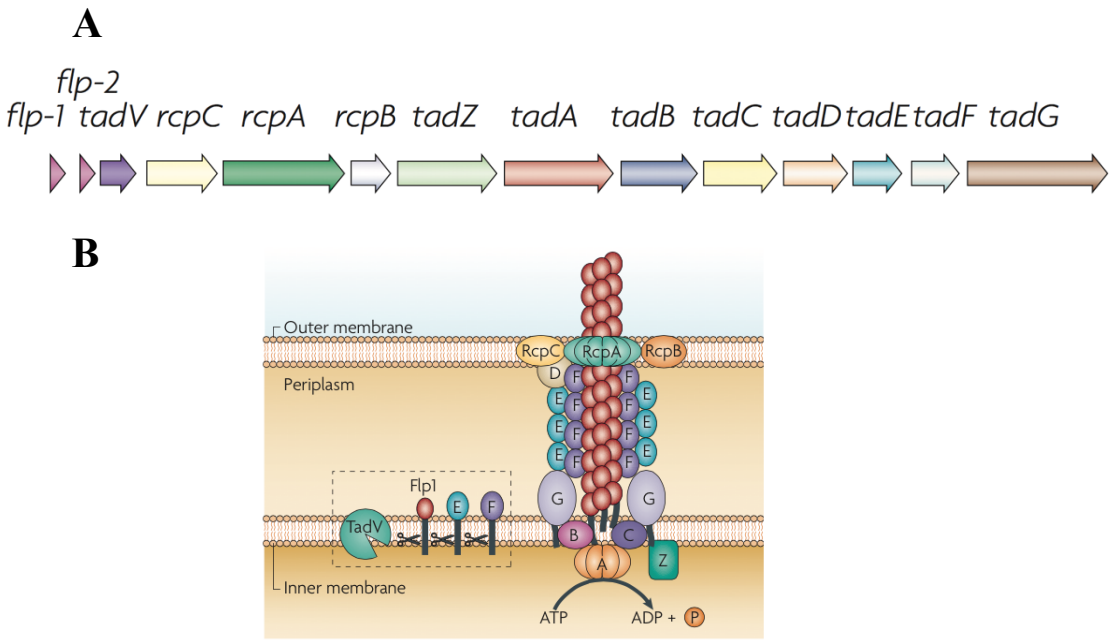

Figure 1.4. Tad locus and fimbriae secretion. (A) A fourteen-gene locus is involved in the biosynthesis and secretion of the fimbriae, which is responsible for the formation of the biofilm and adhesion to host tissues. (B) Eleven genes in this locus encode proteins that form a secretion system spanning the cell envelope to allow for the secretion of the fimbriae. Detailed roles of each protein are described in the text. Image adapted from Tomich, M. et al, *Nature Reviews Microbiology* 2007. 5:363-375.

The next genes encode the secretion system of the fimbriae. *rcpC* encodes an inner membrane protein that is suggested to be involved in the post-translational modification of Flp1 [54, 55]. *rcpA* encodes a protein forming an outer membrane pore that allows for the secretion of the assembled fimbriae [55]. *tadA* encodes a protein that has homology to an ATPase and it provides the energy for the secretion and biosynthesis of the fimbriae [53, 55]. TadE and TadF are called pseudopilins and their leader peptides are also cleaved

by the prepilin peptidase TadV [54, 55]. Mature TadE and TadF together form a channel structure spanning the periplasm to allow for the secretion of the fimbriae [55]. The other genes within the Tad locus, although their specific functions are unknown, are still required for the biosynthesis and secretion of the fimbriae as demonstrated by mutagenic analyses [55, 56].

During laboratory practice, the production of fimbriae is irreversibly lost after repeated culture in liquid media and these bacteria become afimbriated strains [47]. The colonies formed by afimbriated strains are smooth and easily disrupted, with the star shaped internal structure absent in the colony [48]. The afimbriated strains of *A. actinomycetemcomitans* grow in suspension in liquid culture and are therefore easier to genetically manipulate.

1.4.2 The role of MorC in fimbriae biosynthesis.

MorC is a 141 kDa protein encoded by a 3.8 kb gene [57]. It is a conserved protein in hundreds of species of Gram-negative bacteria, including *H. influenzae, E. coli and P. aeruginosa* [57]. The mutation of *morC* in *A. actinomycetemcomitans* leads to multiple phenotypical changes, including decreased membrane stability, decreased leukotoxin secretion and morphological change of the outer membrane [57]. Interestingly, *morC* mutation was found to result in a decreased level of expression of the genes in the Tad locus as predicted by a quantitative proteomics analysis [58]. The decreased fimbriae expression in the *morC* mutant strain was subsequently verified by tagging the fimbrial subunit with a T7 tag followed by ELISA detection using an anti-T7 monoclonal antibody (Personal communication, Mintz, K. P.). The detailed mechanisms of MorC in maintaining the optimal fimbriae biosynthesis and secretion remain under investigation.

1.5. Extracellular matrix protein adhesin A (EmaA)

EmaA is a non-fimbrial adhesin that mediates the binding of *A. actinomycetemcomitans* to collagen, the most abundant protein in the human body [59]. EmaA is the only known adhesin that has specific collagen binding activity in *A. actinomycetemcomitans*, and it is one of the few adhesins identified in oral bacteria that binds to extracellular matrix [60]. EmaA was first identified from a transposon library screen, in which mutants showed decreased collagen binding activity [59]. Subsequent genotypic analysis of the mutants confirmed that the transposon landed in a 6.5 kb gene encoding a 202 kDa protein [59]. EmaA is an autotransporter protein secreted by Type V secretion system, and the secretion is dependent on chaperones including SecB and DnaK [61].

The EmaA molecule is composed of an N-terminal signal sequence, a passenger domain and a C-terminal membrane anchor domain (Figure 1.5). The signal sequence is composed of 56 amino acids, dramatically longer than the average size (5 – 30 amino acids) of signal peptides [62]. The residues within the signal sequence are associated with the optimal secretion of EmaA, as signal sequences containing internal deletions lead to decreased membrane localization of EmaA at 37 °C or higher temperatures and decreased collagen binding activity [62]. The passenger domain is divided into a functional domain and a long stalk domain. The functional domain, which consists of amino acids 70 – 384, forms an ellipsoidal structure and confers collagen binding activity [63]. The stalk domain supports the functional domain and contains multiple coiled-coil sequences for the oligomerization of the monomers [64]. There are seven neck sequences present in the passenger domain, which stabilize the molecule and provide transitions between each

segment [63]. The membrane anchor domain contains β-barrels that form a pore upon oligomerization on the outer membrane of the bacteria for the translocation of the passenger domain. The trimerized EmaA molecules form antennae like structures extending ~100 nm from the surface of the bacteria [64].

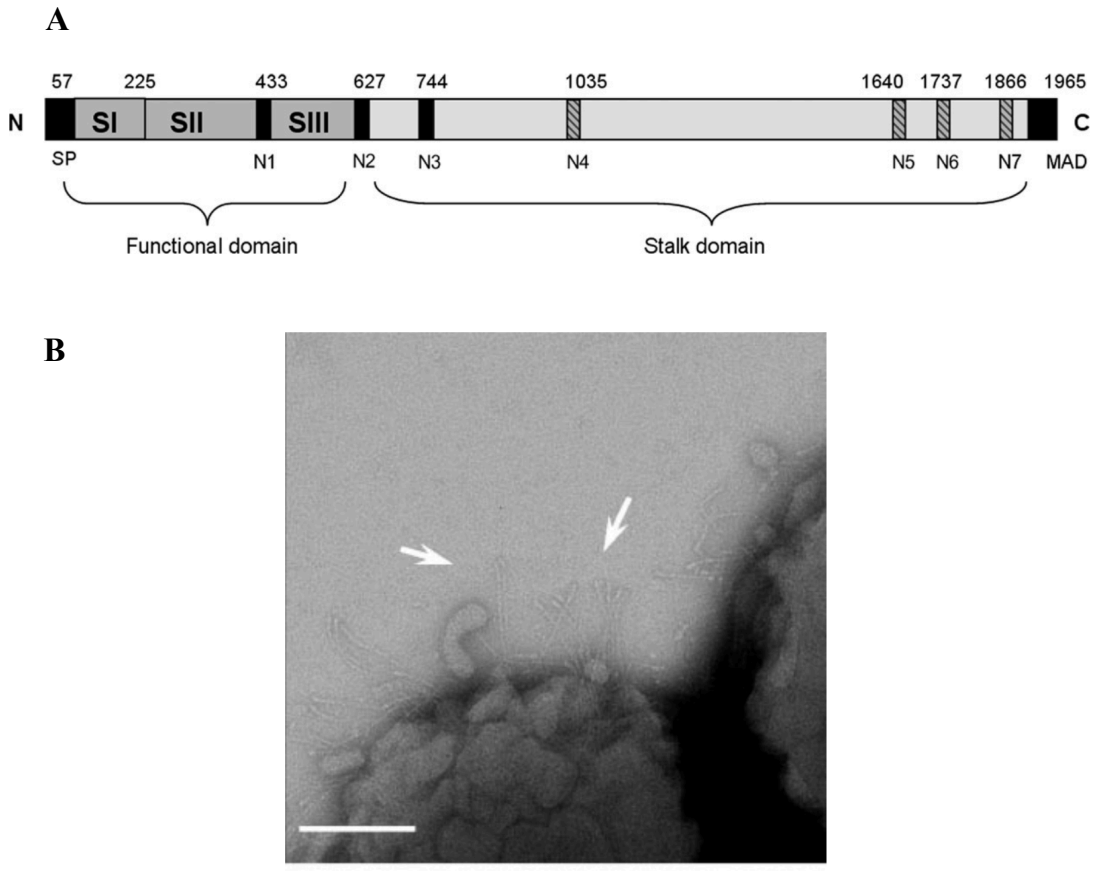

Figure 1.5. Schematic diagram and electron micrograph of EmaA. (A) Schematic model of the EmaA molecule. The N-terminal functional domain is subdivided into three subdomains (SI-SIII). Seven neck sequences (N1-N7) are identified within the passenger domain. SP: signal peptide, MAD: membrane anchor domain. (B) EmaA structures (white arrow) are identified on the surface of *A. actinomycetemcomitans*. Scale bar: 100 nm. Images adapted from Ruiz, T. et al. (2006). *Infect. Immun.* 74:6163-70.

1.5.1. Collagen binding activity

Collagens are the structural components of connective tissues, including tendon, skin and ligaments [65]. Collagens are also the most abundant protein in the human body,

including periodontal tissues and heart valves [65]. In oral tissues, collagens are synthesized by fibroblasts and lie under the epithelium layer. Fibrillar collagens, including collagen type I, II, III, and V are substrates for the binding of EmaA, with type V collagen having the highest affinity [66, 67]. EmaA is able to bind to collagen both *in vitro* and *in vivo* [59, 60]. The collagen binding activity of EmaA was first identified using acid solubilized collagen as substrate in an enzyme-linked immunosorbent assay [59]. An *emaA* mutant shows at least 50% to 60% reduction in binding to solubilized collagen compared to the wild type strain [64]. This binding deficiency is recovered by complementation with the *emaA* gene *in trans*, confirming the role of EmaA in collagen binding *in vitro* [59].

The binding of EmaA to native collagen was demonstrated both *ex vivo* and *in vivo* using either isolated heart valves or an infective endocarditis model [60]. Both wild type and *emaA* mutant strains bind heart valves at a comparably low level when the endothelium remains intact [60]. However, if the endothelium is disrupted by trypsin treatment (*ex vivo*) or catheterization (*in vivo*), wild type *A. actinomycetemcomitans* binds avidly to the decellularized rabbit heart valves. In contrast, the binding of the *emaA* mutant is five fold less and ten fold less compared to the wild type in *ex vivo* and *in vivo* conditions, respectively [60]. These analyses collectively suggest the role of EmaA as a virulence determinant for the initiation of infective endocarditis and many other infections.

The functional domain of EmaA is predicted to be in the N-terminal region of this molecule. A series of mutants in this region has been constructed and introduced into the *emaA* mutant strain to determine the collagen binding domain of the EmaA [63]. The

collagen binding domain is mapped to amino acids 70-384, where multiple collagen binding motifs are present [63]. The *emaA* mutants that contain internal deletions in this region still produce surface structures, but lack the collagen binding activity, suggesting that this domain is involved in collagen binding, but not the secretion or oligomerization of the protein [54]. The mutation of a single amino acid, G162S, leads to a complete loss of the collagen binding activity and the ellipsoidal head structure [63]. Interestingly, the mutation of the same glycine to alanine does not affect the collagen binding activity, suggesting that hydrophobicity of the amino acid in the collagen binding motif is important for the activity of EmaA [63].

1.5.2. Serotype association of EmaA

The genotype of *emaA* is associated with the serotype of the bacteria [68]. The serotype b and serotype c strains encode a full-length EmaA, with a 6.5 kb gene encoding a 202 kDa protein containing 1965 amino acids [68]. The serotype d and a third of serotype a strains tested encode an intermediate form of EmaA, which contains a 279 amino acid in-frame deletion in the stalk domain [68]. The intermediate form of EmaA is suggested to be shorter than the full-length counterpart, possibly due to this internal deletion. Interestingly, the intermediate form of EmaA retains the *in vitro* collagen binding activity, as determined by a 3D matrigel assay [68]. The remaining sequenced serotype a, serotype e and f strains contain premature stop codons in the coding region of *emaA*, which lead to incomplete synthesis of the membrane anchor domain [68]. Since the membrane anchor domain is required for the translocation of the passenger domain and the membrane location of the molecule, these strains do not express EmaA on the bacterial surface and do not bind to collagen [68].

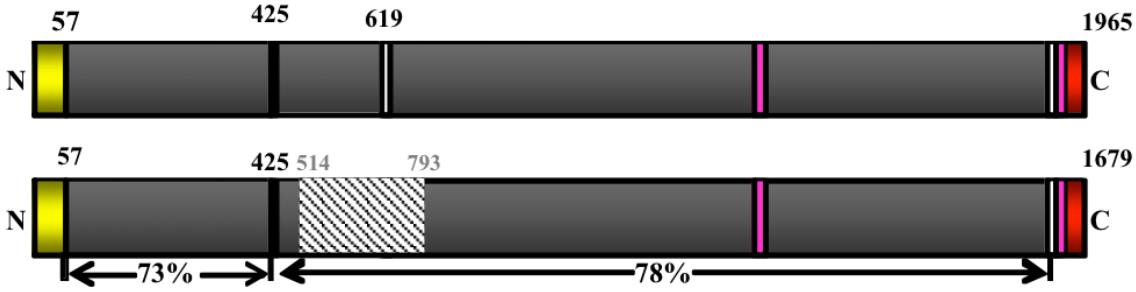

Figure 1.6. Schematic diagram of EmaA molecular forms. Up: Full-length EmaA, bottom: intermediate EmaA. Yellow: signal sequence; white: neck sequence; pink: coiled-coil domain; red: membrane anchor domain. Dashed region correlates to a 279 amino acid internal deletion in the intermediate form of EmaA. The percentiles indicate the sequence homology of the intermediate EmaA to the full-length counterpart.

1.5.3. Glycosylation of EmaA

EmaA is found to be glycosylated and the glycosylation process is associated with the LPS biosynthesis pathway [70]. The role of LPS biosynthesis in EmaA glycosylation was revealed by both genetic and pharmacological analyses [70]. The O-PS of the serotype b *A. actinomycetemcomitans* is composed of repeating L-rhamnose and D-fucose with N-acetyl-D-galactosamine attached to the L-rhamnose. *rmlC* is the gene encoding a TDP-rhamnose 3,5-epimerase, an enzyme required for L-rhamnose biosynthesis. The mutation of *rmlC* leads to decreased molecular weight of EmaA and the loss of reactivity to a rhamnose binding lectin, suggesting the mutation of *rmlC* leads to the loss of EmaA glycosylation. Complementation of *rmlC in trans* restored the phenotypes of EmaA. Mutation of the genes encoding the ATP-binding cassette (ABC) transporter mediating the export of LPS (wzt) and the O-PS ligase (waaL) that links the O-PS to the Lipid A core also lead to the same phenotype, suggesting the role of LPS biosynthesis pathway in EmaA glycosylation [70].

The role of LPS biosynthesis in EmaA glycosylation is further confirmed by pharmacological analyses [70]. Bacitracin is an antibiotic that disrupts the synthesis of the lipid carrier of the O-PS. Bacitracin treatment of the wild type *A. actinomycetemcomitans* leads to a decreased molecular weight of EmaA. This molecular weight decrease is reversed by growth of the bacitracin-treated bacteria in fresh media without bacitracin. These analyses collectively demonstrated the role of LPS biosynthesis in EmaA glycosylation [70]. This glycosylation mechanism appears to be unique to EmaA, as another autotransporter adhesin, ApiA, expressed by *A. actinomycetemcomitans* is not affected by mutation of the *rmlC* gene [70].

Glycosylation is important for the protein structure as it facilitates the correct folding, resistance to enzyme degradation and ensures the activity of the modified protein [72-74, 79-81]. The glycosylation of EmaA is required for the stability and function. Surface exposed EmaA are decreased up to 50% in the glycosylation deficient strains compared to the wild type, whereas the mRNA level of *emaA* is not changed [71]. Therefore, decreased production of EmaA is not due to a decrease in the transcription of the gene [71]. The collagen binding activity of the EmaA from the mutant is also decreased, as revealed by the collagen binding assay. Overexpressing *emaA* in the mutant strain does not complement the collagen binding defect, suggesting that the decreased collagen binding activity is not due to a decreased protein expression level [71].

1.6. Periodontitis and immune response

1.6.1 The innate immune system

The innate immune system is the first line of defense of the body against pathogens [74]. Innate immunity occurs early (0-4 hours after infection) upon pathogen invasion

and operates with less specificity compared to adaptive immunity, which is more specific and longer-lasting [74]. The outermost barrier of the innate immune system is the epithelial layer, which secretes antimicrobial peptides (defensins, cathelicidins, protegrins) and antimicrobial enzymes (lysozymes, secretory phospholipase A_2) [74].

The complement system is also a major component of innate immunity. The complement system mediates the direct killing of pathogens by forming membrane attack complexes on the surface of the bacteria, which subsequently leads to the lysis of the bacteria. Some fragments of the complement molecules, such as C3a and C5a, are efficient inducers of the inflammatory response [74]. The complement molecules also bind to the surface of the bacteria and this opsonization induces engulfment by phagocytic cells. Immune cells involved in phagocytosis of pathogens include neutrophils, macrophages and dendritic cells [74]. These cells recognize and capture pathogens through phagocytosis, macropinocytosis and receptor-mediated endocytosis using C-type lectin receptors and Fc receptors [89]. When the pathogens are taken up into the phagosomes, lysosomes are directed and fused with the phagosomes, forming phagolysosomes. The pathogens are degraded in the phagolysosome, processed to antigens, and subsequently presented to T cells in MHC complexes [76].

1.6.2 Dendritic cells

Dendritic cells are a group of professional antigen presenting cells playing a key role in the innate immune system [76]. Dendritic cells were so named because of their branched processes that resemble the dendrites of neurons. Dendritic cells have the ability to open the tight junctions of the epithelial layer and send dendrites out to sample the environment, thus playing a sentinel role in the immune system [77]. Immature or

inactivated dendritic cells have a high capacity for capturing particles. After antigen uptake, the dendritic cells become mature, with a decreased phagocytic capability and increased expression of MHC-II-antigen complexes and co-stimulatory molecules on the surface [76]. The mature cells migrate to secondary lymphoid organs, where they present the antigens to antigen-specific T lymphocytes, mainly CD4+ T cells, to initiate the adaptive immune response [76]. In addition, dendritic cells also express MHC-I molecules on the surface and present antigens to CD8+ T cells by cross-presentation. Therefore, dendritic cells are a major cell type that bridge the gap between the innate and adaptive immune responses [78].

1.6.3. The interaction of dendritic cells with bacteria

Multiple bacteria, including obligate and facultative intracellular pathogens, are internalized and survive inside dendritic cells. These bacteria include *Mycobacterium tuberculosis, Listeria monocytogenes*, *Bordetella bronchiseptica* and *Porphyromonas gingivalis* [83-87]. The recognition of pathogens is accomplished by a group of receptors called pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) [79]. Among these PRRs, the capture of the pathogen is conducted by a group of C-type lectin receptors [88]. These receptors have carbohydrate recognition domains that interact with the glycan moieties on the pathogens. Some well characterized C-type lectins include Langerin, Dectin-1 and Dendritic cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN) [89].

Internalized bacteria have evolved various mechanisms to survive inside dendritic cells and delay and/or decrease the level of the antigen presenting process [94]. These mechanisms include the secretion of a series of effector molecules through type III and

Type IV secretion systems to the host cell, to prevent or delay the fusion between the phagosome and lysosome and to manipulate the cytoskeleton of the host [90]. For example, *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), a facultative intracellular bacterium associated with gastrointestinal infections, secrete multiple virulence factors that are encoded on a gene locus called Salmonella pathogenicity island 2. These effector molecules are delivered from the Salmonella containing vacuole to the host cell by a type III secretion system [94]. These effector molecules cooperate to subvert host cell singaling and prevent the fusion of the lysosome to the phagosome, which disrupts the antigen processing and contributes to its immune evasion. Thus, the bacteria utilize the dendritic cells as vehicles or "Trojan horses" for their survival, replication and tissue dissemination to the non-oral tissues while maintaining evasion from the host immune systems [90].

Listeria monocytogenes, a bacterium involved in food borne illness, is found to enter dendritic cells and persist inside dendritic cells for at least 24 hours [95]. Those intracellular bacteria are able to manipulate the host cell cytoskeleton to form actin polymers to facilitate their intracellular movement and cell-to-cell dissemination [95]. In contrast to what was found in *Salmonella*, *L. monocytogenes* strains that lack the production of some virulence factors such as listeriolysin O have a better survival rate inside dendritic cells compared to the wild-type counterpart [95]. This finding is in correlation with the fact that *L. monocytogenes* is able to survive and replicate in the phagosome, thus preventing the immune recognition and the subsequent responses of the host cells.

Bordetella bronchiseptica, a Gram-negative bacteria associated with respiratory infections, is found to be able to be internalized into dendritic cells and survive in dendritic cells for at least 72 hours [96]. Interestingly, the results suggest that *B. bronchiseptica* utilize multiple adhesins, including those encoded by the *bvg* locus and those that are independent of the locus, to interact with different receptors on dendritic cells [96].

According to the previous research, *A. actinomycetemcomitans* is not able to survive in the other two groups of phagocytic cells in the innate immune system, i.e. neutrophils and macrophages [90, 91]. However, the interaction of *A. actinomycetemcomitans* with dendritic cells had not been characterized. In this research, we used a dendritic cell internalization assay to investigate the interaction of this bacterium with bone marrow derived dendritic cells. *A. actinomycetemcomitans* was found to be internalized into dendritic cells and survived within dendritic cells. Furthermore, a surface glycoprotein EmaA was found to play an important role in the interaction and/or the survival of the bacterium inside dendritic cells.

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CHAPTER 2: RESEARCH DESIGN AND RESULTS

2.1. Materials and methods

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this research are listed in Table 1 (Page 46) and Table 2 (Page 48), respectively. All *A. actinomycetemcomitans* strains were streaked onto TSBYE plates (3% Trypticase soy broth, 0.6% Yeast extract, 1.5% agar; Beckton Dickinson, Franklin Lakes, NJ) from frozen stocks and grown to a confluent biofilm (36 - 48 h) at 37°C in a humidified 10% CO_2 atmosphere. Antibiotics were added to a final concentration of 100 μg/mL ampicillin, 50 μg/mL kanamycin and 50 μg/mL spectinomycin, when necessary. Unless mentioned elsewhere, all *Escherichia coli* strains were grown with agitation at 37°C in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract and 0.5% NaCl; Becton Dickson, Franklin Lakes, NJ) containing 100 μg/mL ampicillin, 50 μg/mL spectinomycin when appropriate.

Construction of the isogenic mutant strains. The *emaA* mutant strain was constructed by allelic replacement via conjugation following the published procedure with modifications [2]. An internal fragment of *emaA* corresponding to nucleotides 80-1285 was amplified and disrupted with the spectinomycin resistance gene *aad9* at nucleotide 609. This disrupted *emaA* fragment was introduced into the EcoRI site of a conjugative vector pVT1460, making the plasmid pKM524. The new plasmid was transformed into the *E. coli* strain KM482 which requires diaminopimelic acid (DAP) for growth to construct the donor strain. The wild type fimbriated clinical isolate VT1257 (serotype b) was used as the recipient strain.

The recipient cells were grown on a TSBYE plate by confluency streaking for 24 hours, collected using a sterile glass slide and resuspended in 1 mL TBSYE. The donor cells were grown in LB broth supplemented with 0.3 mM DAP until an OD_{600} = 0.3. One milliliter of donor cells were then collected, washed once with TSBYE and resuspended in 250 μL TBSYE. The recipient cells and donor cells were mixed in a 2.5:1 ratio and the conjugation mixture was spotted onto a TSBYE plate supplemented with 0.3 mM DAP. The plate was incubated for 15 min at room temperature, followed by 5 h incubation in a humidified 10% CO_2 atmosphere at 37°C to allow for the conjugation. Following the incubation, 1 mL of TSBYE was added to the plate and the cells were collected with a sterile glass slide, serial diluted, spread onto TSBYE plates containing 50 μg/mL spectinomycin and incubated for 72 h.

The spectinomycin resistant colonies were replica-plated onto TSBYE plates containing either 100 μg/mL kanamycin or 50 μg/mL spectinomycin. The transconjugants that were resistant to spectinomycin but sensitive to kanamycin were tested for the correct integration of the *aad9* gene into the chromosome by PCR. Genomic DNA from the transconjugants was isolated using Puregene DNA extraction Kit (Gentra Systems, Minneapolis, MN). The correct integration of the *aad9* gene was verified by PCR using the primers: CBP1-UpF: 5'-ACATGCATGCAACAAATCGCCGTC-ATCGCC-3' and Spc-2R: 5'-CTCTTGCCAGTCACGTTACG-3'.

To confirm the unique role of EmaA in dendritic cell internalization, an isogenic mutant for *morC* was generated using the same protocol as described above using the following plasmid construct. The *morC* mutation construct contains 1 kb DNA fragment

upstream and 1 kb fragment downstream of the *morC* gene, with the spectinomycin resistance gene *aad9* in between. The mutation construct was ligated with the mobilizable plasmid pVT1460 to make the plasmid pKM551. The *E. coli* strain KM482 was transformed with pKM551 to construct the donor strain. The wild type *A. actinomycetemcomitans* VT1257 was used as the recipient strain. The conjugation was conducted as described above and the genotype of the mutant was verified by PCR using the following primers: Omp67F: 5'-TCTGGACGTATTGCTTTATCCGC-3' and Spc-2R: 5'-CTCTTGCCAGTCACGTTACG-3'.

Complementation of the *emaA* mutant strain. The complementation of the *emaA* mutant was performed by transforming pKM9, a shuttle plasmid containing the complete sequence of the full-length *emaA* and 500 bp of the upstream region, into the *emaA* mutant strain by electroporation as described [4]. The transformants were selected on TBSYE-agar plates containing 100 μg/mL kanamycin and the presence of the plasmid pKM9 was verified by colony PCR using the following primers: pKM1F: 5'-CTTTATGCTTCCGGCTCGTA-3' and CBP1-1259R: 5'- GGAGAGAAAT-AATAACGGGGC - 3'.

Construction of the GFP-expression strains. *A. actinomycetemcomitans* strains VT1257, KM696, KM700 were transformed with a broad-host plasmid harboring a gene encoding the green fluorescence protein (GFP) of *Aequorea victoria* (pKM542) for confocal laser scanning microscopy and fluorescence activated cell sorting (FACS) analyses [3]. Briefly, *A. actinomycetemcomitans* strains were grown on TSBYE plates (antibiotics added as appropriate) until confluency (24 - 36 h). Two plates of bacteria were scraped from the plates with a sterile glass slide and washed in electroporation

buffer (0.57 mM KH_2PO_4, 2.43 mM K_2HPO_4 and 272 mM sucrose in 15% (v/v) glycerol) three times at 4°C. The cell suspension was then mixed with 0.5 μg plasmid and the electroporation was performed at 2.5 kV. The bacteria were recovered in 1 mL TSBYE broth at 37°C for 4 h and spread onto TSBYE plates containing 100 μg/mL of ampicillin for the selection of the transformants. The plates were incubated for 3 days and the transformants were verified by the expression of green fluorescence using a fluorescent microscope.

Immunoblotting analyses. The *emaA* mutant and the *morC* mutant were further characterized by immunoblotting to confirm the lack of expression of the proteins of interest. Bacterial strains were grown in 100 mL TSBYE in T175 tissue culture flasks in at 37°C in a humidified 10% CO_2 atmosphere for 16 hours. Spectinomycin was added to a final concentration of 50 μg/mL for the maintenance of the mutant. The cells were scraped from the surface of the culture flasks, suspended in PBS and lysed using a French pressure mini cell at 18,000 psi for three times at 4°C. The cell lysate was clarified by centrifugation at 12,000 × g to remove the intact cells. Membranes fractions were collected by centrifugation at 100,000 × g at 4°C. The pellet containing the whole membrane fraction was resuspended with 250 μL PBS and the protein concentration of the suspension was determined by BCA Protein Assay Kit following the manufacturer's protocol (Pierce) with bovine serum albumin as the standard.

For the detection of EmaA, 250 μg protein of each sample was separated in a 5% polyacrylamide-SDS gel at 40 V for 3 h. For the detection of MorC, 250 μg protein of each sample was separated in a 4-15% gradient polyacrylamide-SDS gel at 80 V for 1.5

h. Proteins were subsequently transferred onto PVDF membranes at 100 mA for 2 h. The membranes were blocked with 5% non-fat milk and incubated with either 15 μg/mL anti-EmaA stalk monoclonal antibody or 1 μg/mL affinity purified anti-MorC antibody at room temperature for 1 h. The membrane was washed vigorously in 1 × TBST (0.5% (v/v) Tween-20 in TBS) for 3 times, 5 min each. The membrane was then probed with horseradish peroxidase (HRP) conjugated goat-anti-mouse secondary antibody for EmaA detection and HRP conjugated goat-anti-rabbit secondary antibody for MorC at room temperature for 1 h. The immune complexes were detected by Pierce West Femto detection reagent.

Dendritic cells culture. Mouse bone marrow derived dendritic cells (mBMDCs) were prepared according to published protocols with modifications [1]. Briefly, hematopoietic cells from mouse bone marrow of freshly isolated mouse femurs were isolated by flushing the bone with PBS containing 2% fetal bovine serum using 1 mL syringe. The cells were separated from debris and the cell aggregates were disrupted by repeated pipetting. The bone marrow hematopoietic cells were grown and differentiated to dendritic cells in complete dendritic cell media (RPMI-1640 supplemented by 10% fetal bovine serum, 0.1 mM sodium pyruvate, 0.1 mM folic acid, 0.1 mM glucose, 50 μM β-mercaptoethanol, 100 U/mL penicillin, 100 U/mL streptomycin and 20 ng/mL granulocyte macrophage – colony stimulating factor (GM-CSF)) at 37°C in a humidified 5% CO_2 atmosphere for 10 days. Conditioned media were removed and 15 mL new media were added into the culture every 2 days. Prior to each experiment, dendritic cells

were collected by centrifugation at 450 × g for 10 min, washed and resuspended with antibiotic-free complete dendritic cell media.

Dendritic cell internalization assay. The internalization assay was performed following published protocols with modifications to study the interaction of *A. actinomycetemcomitans* with dendritic cells [5, 6]. Dendritic cells (3×10^5) were seeded into each well of 12-well plates in 1 mL antibiotic-free complete dendritic cell media. Bacterial strains were grown on agar plates (for *A. actinomycetemcomitans*, antibiotics added as appropriate), or in LB broth containing 100 μg/mL ampicillin (for *E. coli* strain KM542). The bacteria were collected, washed once with 1 × phosphate buffered saline (PBS) and resuspended in antibiotic-free complete dendritic cell media.

Bacterial input of each strain was normalized to protein concentration to ensure equivalent inoculum to the dendritic cells. Bacteria corresponding to 40 μg total protein were inoculated into each well and co-cultured with dendritic cells for 2 h in a humidified 5% CO_2 atmosphere at 37°C to allow for the internalization. Gentamicin (100 μg/mL) was subsequently added to the co-culture system for the remainder of the assay to kill all the extracellular bacteria.

Confocal laser scanning microscopy. The internalized bacteria in dendritic cells were examined by confocal laser scanning microscopy. The dendritic cells were grown on sterile glass coverslips and co-cultured with GFP-labeled bacteria. The dendritic cells with or without the bacteria were stained with 10 μM Hoechst 33324 and Mitochondria-Red (Invitrogen) to label the nuclei and mitochondria, respectively. The stained dendritic cells were then washed with PBS and fixed with 2.5% formaldehyde (Ted Pella, Redding,

CA). The cell samples were imaged using an LSM 510 Meta confocal laser scanning microscope (Zeiss, Goettingen, Germany), attached to an Axiovert 200 M inverted light microscope (Zeiss) using a 63× oil immersion objective lens. The internalized bacteria were visualized by the green fluorescence produced by the GFP. For live imaging, dendritic cells co-cultured with GFP-labeled bacteria were mounted into a heated chamber with humidified 5% CO_2 atmosphere. Images were taken at 1 min intervals and overlaid with phase contrast images.

Flow cytometry. Approximately 1×10^5 dendritic cells were transferred into tubes, washed once with PBS and fixed with 2.5% paraformaldehyde. The fluorescent cells were then analyzed using a BD LSR II flow cytometer (Becton Dickinson) with a single excitation wavelength (488 nm) and band filters for FITC (525 nm). The cell population was classified for size (forward scatter) and granularity (side scatter) and 5,000 cells were evaluated per condition. Dendritic cells treated with an actin polymerization inhibitor, Cytochalasin D (5 μg/mL), were included in the experiment to determine the role of actin polymerization in bacteria internalization.

Live bacteria recovery. Dendritic cells co-cultured with bacteria corresponding to 40 μg of total protein for 3 h were collected by centrifugation at 450 × g for 10 min and lysed with dendritic cell lysis buffer (0.5% (v/v) Triton X-100 in PBS). The cell lysates were serial diluted with TSBYE and spread onto TBSYE plates to recover the live internalized bacteria. The plates were incubated at 37°C in humidified 10% CO_2 for 48 – 72 h and the colonies on each plate were enumerated [6].

Statistical analysis. All the analyses were performed in duplicates in this study and repeated a minimum of 2 times. Results are presented as mean ± standard deviation. Student's t-test was used for the data analysis and a p-value < 0.01 was used as the threshold for statistical significance.

2.2. Results

Detection of EmaA and MorC by immunoblotting. For the detection of EmaA and MorC, we used afimbriated wild type *A. actinomycetemcomitans* and respective isogenic mutants as controls. The wild type of both afimbriated strain and fimbriated strain showed strong immune reactivity in the immunoblotting for both EmaA and MorC (Figure 2.1, 2.2). The mutant strains showed no visible detection of the proteins of interest. These data collectively showed at protein level that the mutants were successfully constructed by allelic replacement.

Confocal laser scanning microscopy of co-cultured dendritic cells. We examined the intracellular localization of the GFP-labeled *A. actinomycetemcomitans* in dendritic cells by laser scanning confocal microscopy. The mitochondria of the dendritic cells were stained with Mitotracker-Red to define the cytoplasm (Figure 2.3) and the nuclei were counterstained with Hoechst 33324 (Figure 2.3). The confocal microscopy images were overlaid with phase contrast images to show the location of the cells (Figure 2.3). Digital images acquired with a 63× oil immersion lens showed fluorescent bacteria inside dendritic cells from the images taken 3 h and 6 h after co-culture (Figure 2.3), suggesting that the bacteria were internalized into dendritic cells within 3 hours.

Live imaging of the dendritic cells co-cultured with *A. actinomycetemcomitans.*

Live imaging of the dendritic cells co-cultured with GFP-labeled VT1257 was performed

with a confocal microscope equipped with a heated stage and a humidified 5% CO_2 chamber. Images of the same field were taken in one-minute intervals. Intracellular fluorescent bacteria were observed in the image corresponding to 30 min post inoculation, suggesting that internalization occurred within 30 minutes (Figure 2.4). The irregular shape of the fluorescence in the host cell indicated that it represented a bacterial aggregate. This agreed with the characteristics of fimbriated strains of *A. actinomycetemcomitans*, which tend to form cell aggregates [10].

Intracellular bacteria from the same dendritic cell were also present in the image corresponding to 60 minutes post inoculation (Figure 2.4). Interestingly, there were two bacterial clumps of bacteria in the 60 min image compared to a single clump in the 30 min image. This suggested that the bacteria survived and possibly replicated inside dendritic cells during that 30 min period. One of the cell aggregates appeared to be migrating to the border between the host cell and the adjacent cell, suggesting it was egressing from the host. These data suggested that this bacterium was able to survive and replicate in dendritic cells. The potential transmission of *A. actinomycetemcomitans* between host cells was also suggested by these images.

Flow cytometry. In order to expand our finding from individual dendritic cell to the whole cell population, dendritic cells co-cultured with GFP-expressing *A. actinomycetemcomitans* or *E. coli* with the same plasmid for 6 h were analyzed by flow cytometry. Consistent with the confocal images, dendritic cells were associated with *A. actinomycetemcomitans* (Figure 2.5). Dendritic cells co-cultured with *E. coli* harboring the same GFP construct displayed a 50% decrease in the fluorescence intensity compared to *A. actinomycetemcomitans*. Considering the phagocytic nature of dendritic cells, a set

of dendritic cells were treated with 5 μg/mL Cytochalasin D, an actin polymerization inhibitor, and co-cultured with the same amount of *A. actinomycetemcomitans* and *E. coli*. The dendritic cells treated with Cytochalasin D displayed a 90% decrease and an 80% decrease in fluorescence intensity for those co-cultured with *A. actinomycetemcomitans* and with *E. coli*, respectively. This difference suggested that actin polymerization of the dendritic cells plays an important role in the uptake of bacteria.

Dendritic cells express multiple C-type lectin receptors, such as Dectin-1, Langerin and DC-SIGN (CD209), for the internalization of pathogens [13]. *A. actinomycetemcomitans* expresses a glycoprotein EmaA as surface structures [14]. This lead us to hypothesize that EmaA binds to C-type lectin receptors via glycoprotein – lectin interaction, which facilitates the internalization into dendritic cells. To test this hypothesis, we constructed an isogenic *emaA* mutant by allelic replacement, labeled it with the same GFP-expression vector and co-cultured it with dendritic cells in parallel with the wild type strain. From our result (Figure 2.6), we found that the dendritic cells co-cultured with *emaA* mutant had ~70% decrease in fluorescence compared to the wild type. This decrease in fluorescent intensity was not observed when the *morC* mutant strain was co-cultured with dendritic cells. These results suggested that EmaA plays an important role in the internalization of *A. actinomycetemcomitans* into dendritic cells.

Live bacteria recovery. We set out to determine the number of live bacteria inside dendritic cells by gentamicin protection assay. We found a 70% decrease of the number of live *emaA* mutant cells from the dendritic cells compared to the wild type cells (Figure 2.7). This decrease was not present when the *morC* mutant strain was added to the cultures (Figure 2.7). These results further supported the hypothesis that *A.*

actinomycetemcomitans survives inside dendritic cells and EmaA has a role in the internalization of *A. actinomycetemcomitans* into dendritic cells.

In order to confirm the role of EmaA in dendritic cell internalization, we complemented the *emaA* mutant *in trans* by introducing pKM9, a shuttle plasmid containing the full-length *emaA* with its endogenous promoter, into the *emaA* mutant strain [15]. The *emaA* complemented strain was included in the gentamicin protection assay. We found that the complementation of *emaA* restored the phenotype of the *emaA* mutant strain (Figure 2.8). This experiment confirmed the role of EmaA in dendritic cell internalization.

The genotype of *emaA* is closely associated with the serotypes of *A. actinomycetemcomitans* [16]. Serotype b and c strains express a full-length EmaA whereas serotype a and d strains express an intermediate form of EmaA with an internal deletion in the stalk domain (Figure 1.5). To determine if the molecular form of EmaA is a requirement for internalization by dendritic cells, strains of *A. actinomycetemcomitans* expressing the intermediate form of EmaA were used in the live bacteria recovery assay. Interestingly, we found an 84% and 91% decrease of the live bacteria recovered from dendritic cells co-cultured with a serotype a and a serotype d strain, respectively (Figure 2.9). The number of live bacteria was less than the *emaA* mutant from the serotype b. This suggests that dendritic cell internalization and survival within *A. actinomycetemcomitans* varies among strains and may depend on the molecular form or the composition of the glycan of EmaA.

Table 1. Bacteria strains used in this study

Strains	Description	References
A. actinomycetemcomitans		
VT1257	Wild type, formerly IDHaas10a, serotype b clinical isolate	IDH‡
KM596	VT1257 transformed with plasmid pKM542	This study
KM696	*emaA* mutant in VT1257, spectinomycin adenyltransferase gene (*aad9*) inserted into a unique HindIII site of *emaA*	This study
KM695	KM696 transformed with plasmid pKM542	This study
KM732	*emaA* mutant complemented with plasmid pKM9	This study
VT1230	Wild type, formerly IDH2303, serotype a clinical isolate	IDH
VT1246	Wild type, formerly IDH3863, serotype d clinical isolate	IDH
KM700	*morC* mutant in VT1257, the *morC* gene is replaced with spectinomycin adenyltransferase gene (*aad9*)	This study
E. coli		
DH10B	*F– mcrA Δ(*mrr-hsd*RMS-mcrBC) Φ80lacZΔ*M15 *Δ*lac*X74 recA1 endA1 araD139 Δ(ara leu) 7697 galU galK rpsL nupG λ–*	Invitrogen, Carlsbad, CA

DH5α (λ pir)	*endA1 hsdR17 (r^-m^+) supE44 thi-1 recA1 gyrA1(Nal^r)relA1D(lac-IZYAargF)U169 deoR (F80 dLacD(lacZ)*M15*) pir* R6K	Invitrogen, Carlsbad, CA
KM9	DH10B transformed with plasmid pKM9	[17]
KM482	(F-) RP4-2-Tc::Mu *Δ*(*dapA*)::(*erm-pir*), Km^r, Em^r	This study
KM524	KM482 transformed with plasmid pKM524	This study
KM542	DH5α transformed with plasmid pKM542	[3]
KM551	pKM482 transformed with plasmid pKM550	In press

‡Institue of Dentistry, Helsinki, Finland.

Table 2. Plasmids used in this study[a]

Plasmids	Description	References
pKM1	Shuttle vector, the plasmid pDMG4 with its spectinomycin adenyltransferase (*aad9*) replaced by the aminoglycoside phosphotransferase gene (*kan*), Km^r	[18]
pKM9	Full length *emaA* with 500bp upstream region in pKM1	[18]
pVT1460	Conjugative vector, a derivative of pGP704, containing the aminoglycoside phosphotransferase gene (*kan*), Km^r	[19]
pKM524	pVT1460 containing the *emaA* knockout construct, Sp^r, Km^r	This study
pKM542	Formerly pNP3, *gfp* gene in pMMB67 under the control of *spc* and *tac* promoters, Ap^r	[3], [17]
pKM551	pVT1460 containing the *morC* knockout construct, Sp^r, Km^r	This study

[a] Ap, ampicillin; Km, Kanamycin; Sp, Spectinomycin

Figure legends

Figure 2.1. Characterization of the *emaA* mutant by immunoblotting. Membrane samples with equal amount of protein (250 μg/mL, determined by BCA assay) from each bacterial strain were loaded onto a 5% SDS-polyacrylamide gel and run at 40 V for 3 h. The proteins were transferred onto PVDF membrane and probed by anti-EmaA stalk monoclonal antibody. (WT, Wild type; Mut, Mutant)

Figure 2.2. Characterization of the *morC* mutant by immunoblotting. Whole cell lysates with equal amount of protein (250 μg/mL, determined by BCA assay) were loaded onto SDS-polyacrylamide gels and run at 80V for 1.5 h. The proteins were transferred onto PVDF membrane and probed with affinity purified anti-MorC polyclonal antibody. (WT, wild type; Mut, Mutant)

Figure 2.3. Confocal microscopy of the dendritic cells co-cultured with *A. actinomycetemcomitans*. Mouse BMDCs were grown in GM-CSF and co-cultured with GFP-labeled *A. actinomycetemcomitans*. After the co-culture, the nuclei of the dendritic cells were stained with Hoechst 33324 and the mitochondria were stained with Mitotracker Red. The dendritic cells were fixed with 2% paraformaldehyde and visualized by confocal microscopy 0 h (a, b, c), 3 h (d, e, f) and 6 h (g, h, i) after the inoculation of the bacteria.

Figure 2.4. Live imaging of dendritic cells co-cultured with *A. actinomycetemcomitans*. Mouse BMDCs co-cultured with GFP-labeled *A. actinomycetemcomitans* were imaged by a live imaging system. Images from the same field were acquired in 1 minute intervals and the images representing 0 min, 15 min, 30

min and 60 min after the inoculation of the bacteria were presented. The expanded view of the images taken at 30 min and 60 min after inoculation were also presented.

Figure 2.5. Flow cytometry of dendritic cells with internalized bacteria. Dendritic cells were either treated with 5 μg/mL Cytochalasin D or left untreated and co-cultured with *A. actinomycetemcomitans* or *E. coli*. Dendritic cells co-cultured with *A. actinomycetemcomitans* were fixed with 2.5% paraformaldehyde and the cells were scanned by flow cytometry according to the fluorescence intensity from the fluorescent bacteria associated with the cells.

Figure 2.6. Flow cytometry of dendritic cells co-cultured with various strains. Equal amount of wild type *A. actinomycetemcomitans*, *emaA* mutant *A. actinomycetemcomitans*, *morC* mutant *A. actinomycetemcomitans* and *E. coli* were labeled with the same GFP expression plasmid and inoculated into dendritic cell culture with the same protein amount. After the co-culture, dendritic cells were fixed with 2.5% paraformaldehyde and scanned by flow cytometry.

Figure 2.7. Live bacteria recovered from dendritic cells. Mouse BMDCs were co-cultured with equal amount of the wild type *A. actinomycetemcomitans, emaA* mutant (*emaA*$^-$) or another mutant for an inner membrane protein (*morC*$^-$). Gentamicin was added into the co-culture system 2 h after the addition of the bacteria. Dendritic cells with internalized bacteria were lysed with 0.5% triton X-100 in PBS and the cell lysate was diluted and spread onto TBSYE plates. The plates were incubated for 72 h to recover the bacteria and the bacterial colonies were enumerated.

Figure 2.8. Live bacteria recovery of the *emaA* complemented strain. The *emaA* mutant was complemented *in trans* by transforming a plasmid harboring the full-length

emaA gene with its endogenous promoter. Equal amount of the wild type, *emaA* mutant and the complemented strain were inoculated into the dendritic cell culture for the gentamicin protection assay.

Figure 2.9. Serotype variation of *A. actinomycetemcomitans* recovered from dendritic cells. Serotype a, b and d strains that correlated to 40 μg of total protein were individually co-cultured with mBMDCs for 3 hours. Dendritic cells were then collected, lysed and the cell lysates were diluted and spread onto TSBYE plates. The plates were incubated for 72 hours and CFUs were enumerated.

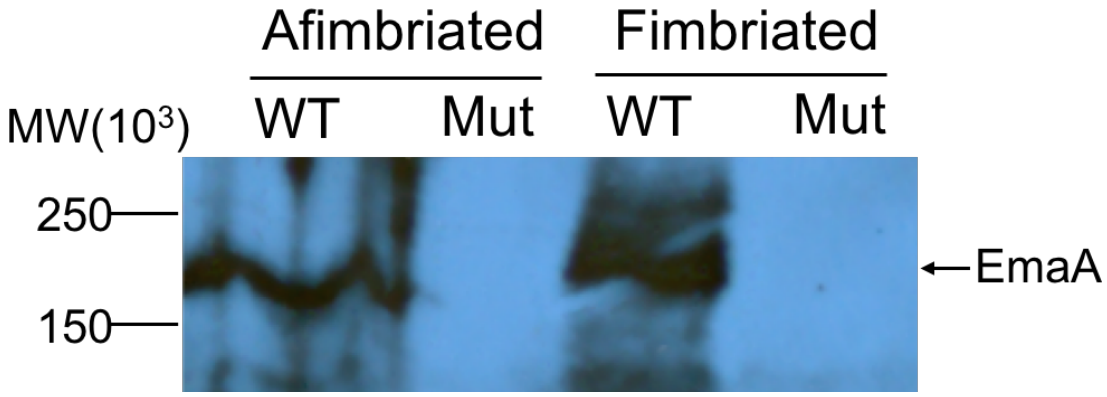

Figure 2.1. Characterization of the *emaA* mutant by immunoblotting.

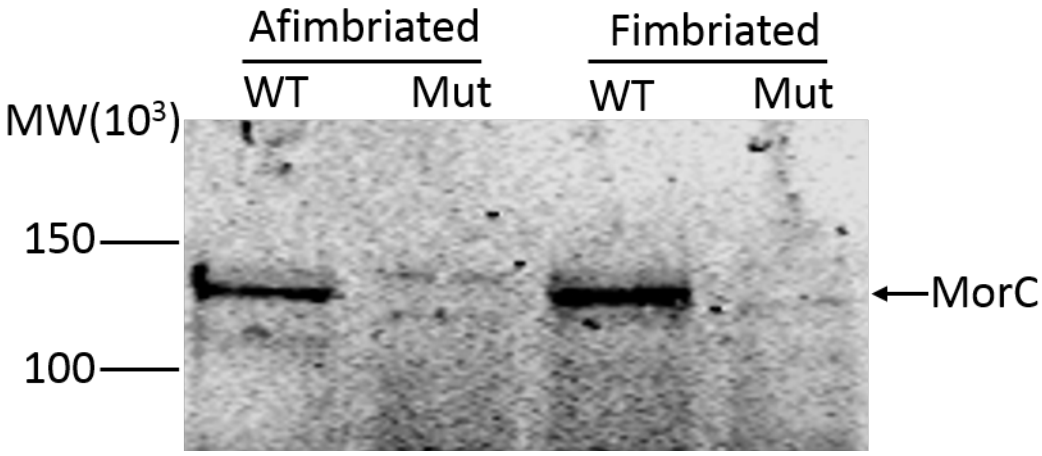

Figure 2.2. Characterization of the *morC* mutant by immunoblotting.

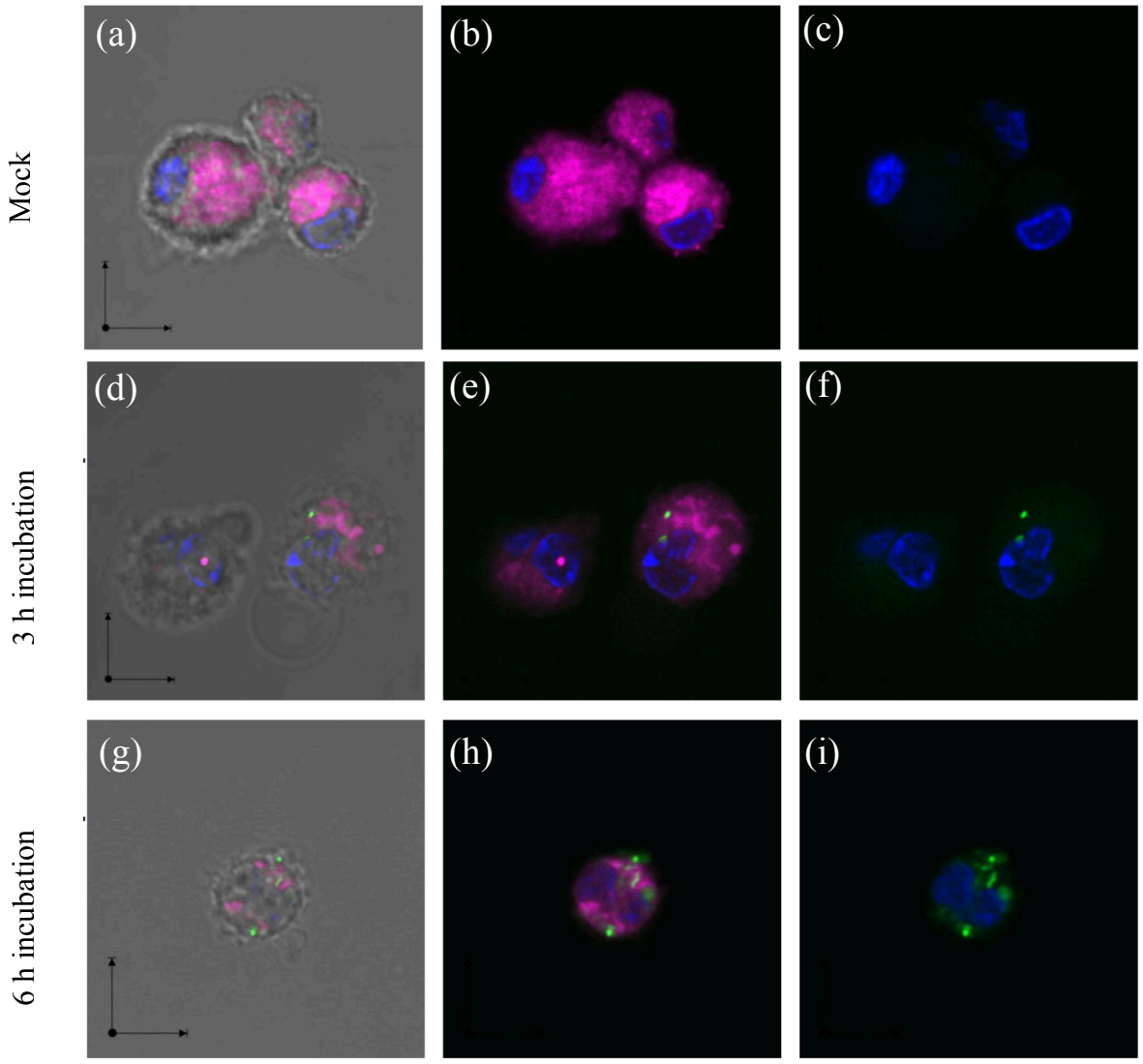

Figure 2.3. Confocal microscopy of the dendritic cells co-cultured with *A. actinomycetemcomitans*.

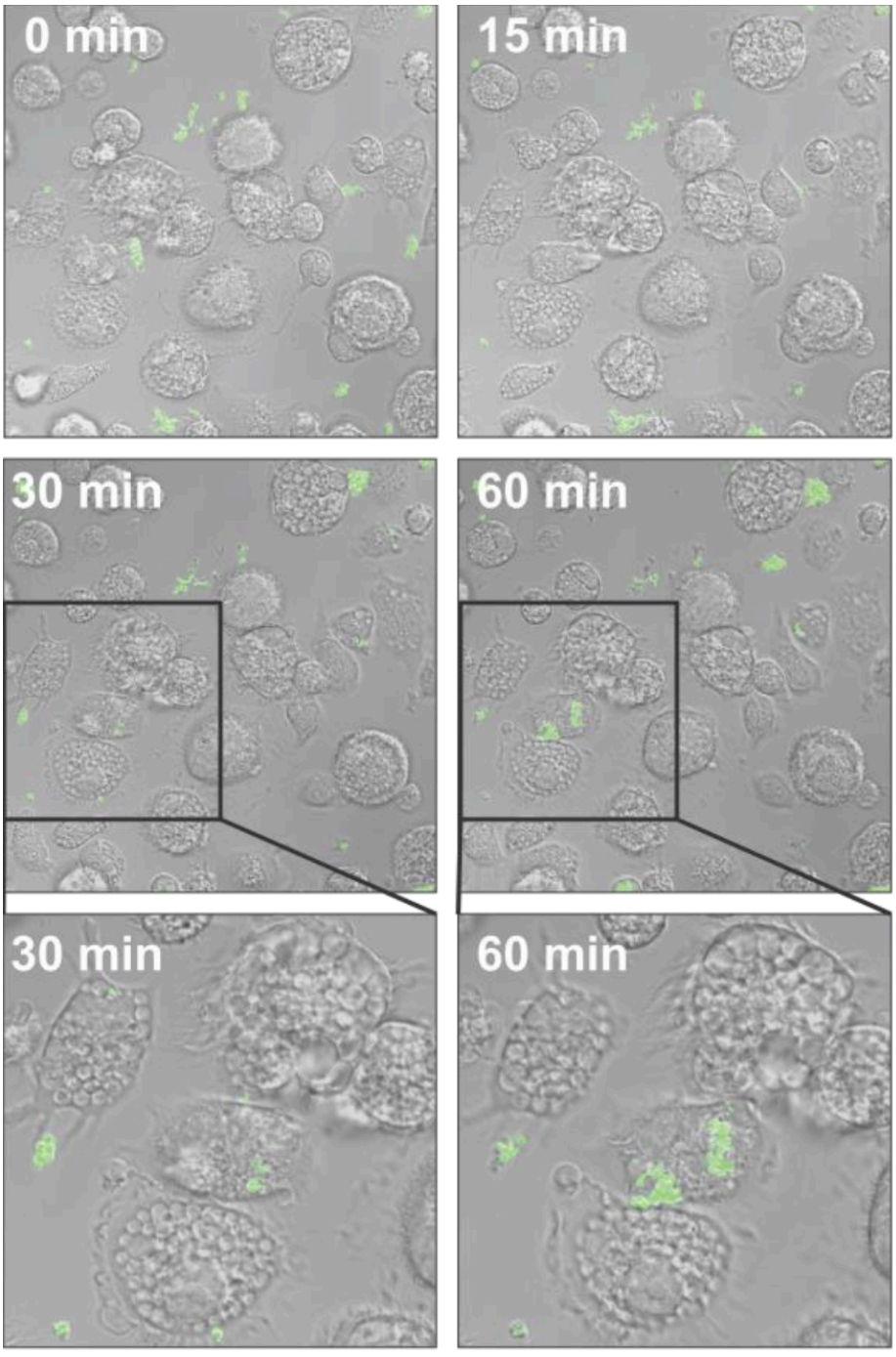

Figure 2.4. Live imaging of dendritic cells co-cultured with *A. actinomycetemcomitans*

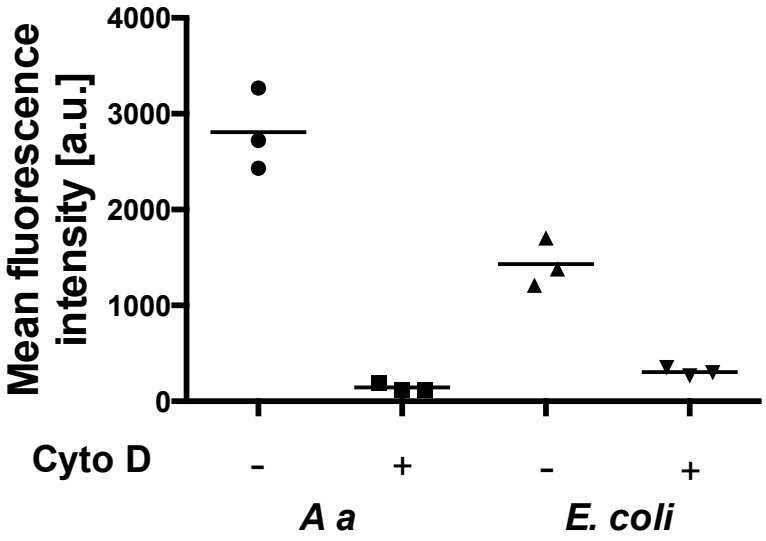

Figure 2.5. Flow cytometry of dendritic cells with internalized bacteria.

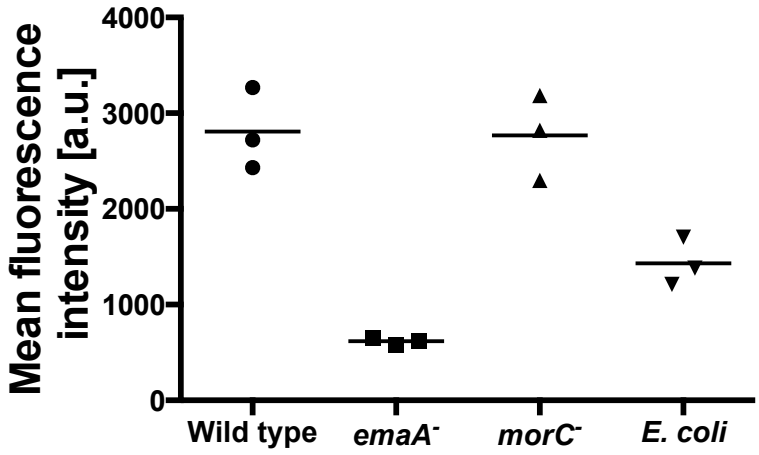

Figure 2.6. Flow cytometry of dendritic cells co-cultured with various strains.

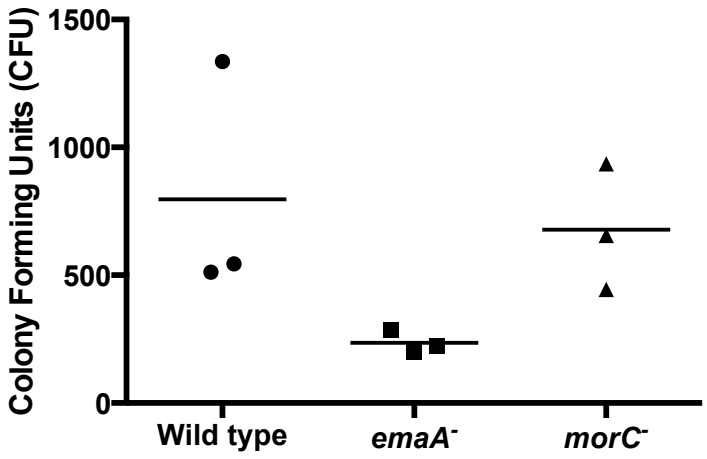

Figure 2.7. Live bacteria recovered from dendritic cells.

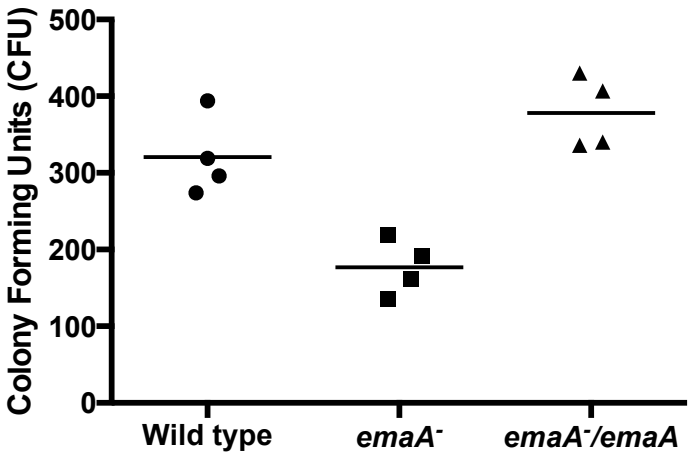

Figure 2.8. Live bacteria recovery of the *emaA* complemented strain.

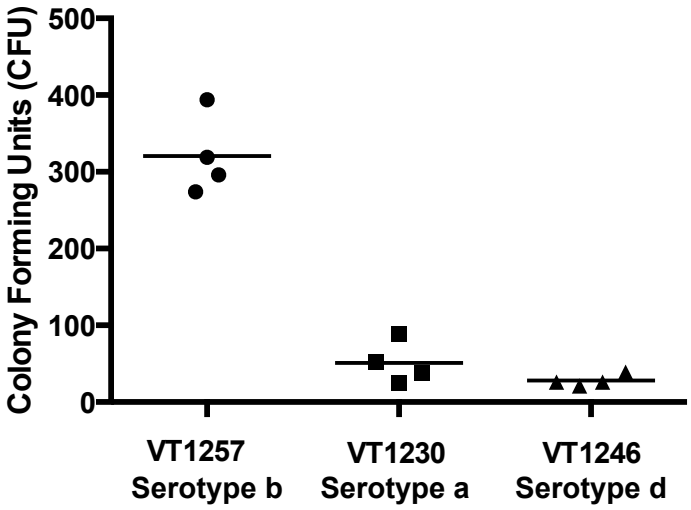

Figure 2.9. Serotype variation of live bacteria recovered from dendritic cells.

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CHAPTER 3: DISCUSSION AND FUTURE DIRECTIONS

3.1. Discussion

In this research, the interaction of the Gram-negative oral pathogen *A. actinomycetemcomitans* with bone marrow-derived dendritic cells was investigated in an *in vitro* internalization assay. GFP-expressing bacteria were co-cultured with dendritic cells and after several hours, *A. actinomycetemcomitans* was found inside dendritic cells and suggested to be replicating (Figure 2.3, 2.4). The internalized bacteria were found in a perinuclear localization in dendritic cells, which suggests that the internalized *A. actinomycetemcomitans* are in vacuoles. Both phagocytosis and receptor mediated endocytosis form membrane bound vacuoles following particle uptake and the content of the vacuoles are destined for degradation along the phagolysosome pathway [1]. These vacuoles mature by membrane fusion events with smaller vacuoles originated from the Golgi and ER, which contain enzymes associated with the degradation of the content of the vacuoles [1]. The ER is contiguous with the nucleus and this may explain the intracellular localization of the bacteria. Furthermore, the absence of randomly distributed bacteria in the cell suggests that *A. actinomycetemcomitans* does not escape from the vacuoles and may usurp the phagolysosome pathway for persistence and survival within the dendritic cells.

Fluorescent bacteria observed inside dendritic cells in the image corresponding to 30 minutes post inoculation suggests that the uptake of the bacteria can be accomplished within 30 minutes (Figure 2.4). Bacterial aggregates were observed within dendritic cells. Based on the known doubling time of *A. actinomycetemcomitans* and the co-culture period, bacterial aggregates appear to be internalized. However, we cannot exclude the

possibility that individual bacteria may also be found in dendritic cells. A study of the interaction of *A. actinomycetemcomitans* with neutrophils suggests that bacterial aggregates are less efficiently internalized compared to single bacterium, possibly due to the large size of the aggregates or the less efficient interaction of bacteria with host cells [6]. Moreover, the auto-aggregation may contribute to the intracellular survival of *A. actinomycetemcomitans* within dendritic cells by protecting the bacteria from the low pH and the enzymes in the phagolysosomes.

Approximately twice the amount of fluorescent *A. actinomycetemcomitans* was associated with dendritic cells compared to *E. coli* when starting with equivalent inoculum (Figure 2.5). This difference does not appear compelling *per se*. However, the significance of this finding is greatly enhanced if the different growth rates of these two organisms are taken into consideration. For a single doubling time of *A. actinomycetemcomitans* (~150 minutes), *E. coli* undergoes 7 divisions (~20 minutes). Therefore, over the life time of the experiment (3 hours), there should be ~8 times the number of *E. coli* associated with dendritic cells than *A. actinomycetemcomitans* if both bacteria were affected to the same extent inside dendritic cells. However, a 50% reduction in the fluorescent intensity of the *E. coli* cells was observed, in sharp contrast to the growth rate of each bacterium. These data suggest that *A. actinomycetemcomitans* survives more efficiently than *E. coli* in dendritic cells.

There are two possible explanations for this observed difference between these two organisms. The first possibility is *A. actinomycetemcomitans* is more robust than *E. coli*. This is highly unlikely as *A. actinomycetemcomitans* is not viable after 4 days in culture whereas *E. coli* survives more than 6 months at 4ºC. The second possibility is *A.*

actinomycetemcomitans has a higher survival capability within dendritic cells compared to *E. coli.* Multiple co-evolved mechanisms, such as delivering effectors molecules to the host cell and interfering the phagolysosome formation, are utilized by bacteria to survive in host cells. The mechanisms used by *A. actinomycetemcomitans* for survival within dendritic cells remain to be investigated.

Dendritic cells, along with neutrophils and macrophages, are professional phagocytic cells playing a key role in the innate immune system [4]. The interaction of *A. actinomycetemcomitans* with neutrophils and macrophages has been investigated in similar *in vitro* internalization assays [5, 6]. *A. actinomycetemcomitans* is rapidly killed after being internalized into neutrophils and macrophages [5, 6]. In contrast, *A. actinomycetemcomitans* appears to persist or survive within dendritic cells. The differences in persistence may be attributed to the different functions and physiologies of the immune cells. Neutrophils and macrophages are very efficient in scavenging and destroying phagocytized particles [7]. Neutrophils are the first group of phagocytic cells that migrate to the infectious foci through chemotaxis followed by macrophages. Following internalization of the pathogens, an oxidative burst is induced generating various toxic compounds such as hydrogen peroxide, superoxide ions and free radicals which kill the internalized microbes [8]. Macrophages also degrade the phagocytized particles by a rapid fusion of the lysosome with the phagosome. In contrast, dendritic cells tend to preserve and accumulate antigens for antigen presentation [7]. In correlation with this unique role, dendritic cells have a lower amount of lysosomal proteases and a lower level of lysosomal proteolysis [8]. These differences may contribute to the alternate intracellular fate of *A. actinomycetemcomitans* in different cells.

The internalization of *A. actinomycetemcomitans* by dendritic cells is mediated by the EmaA structures. Mutational inactivation of *emaA* results in a 70% decrease of bacteria observed in dendritic cells compared to the wild type, which can be restored by *in trans* complementation (Figure 2.8). We hypothesize that the EmaA structures interact with receptors on the dendritic cell surface and the bacteria are taken up in vacuoles. The mutation of *emaA* did not completely abolish the internalization, indicating that other bacterial surface proteins may be involved and that the bacteria are also internalized through non-specific uptake by phagocytosis.

Dendritic cells express multiple C-type lectin receptors for the internalization of bacteria. A Gram-negative oral pathogen, *Porphyromonas gingivalis,* is internalized into dendritic cells by its minor fimbriae [11]. The minor fimbriae are composed of repeating 67 kDa protein monomers Mfa1 [11]. Mfa1 is found to be glycosylated and the carbohydrate moiety contains fucose, mannose, N-acetylglucosamine and N-acetylgalactosamine, which are known ligands for the lectin receptor, Dendritic cell-specific ICAM3-grabbing nonintegrin (DC-SIGN) [12]. Interestingly, EmaA is also a glycoprotein and we hypothesize that the glycan moiety by itself or in combination with the protein structure is required for the interaction with the C-type lectin receptors on the surface of dendritic cells. The glycan moiety of EmaA is suggested to be composed of sugars associated with the O-PS of the LPS, which includes L-rhamnose, D-fucose and N-acetyl-D-galactosamine [14]. Two of the three sugars are also ligands for DC-SIGN. These findings suggest that the EmaA expressed by serotype b strains may interact with DC-SIGN. On the other hand, dendritic cells also express other lectin receptors, including Dectin-1 and Langerin. Whether EmaA binds to one or multiple specific receptors to

facilitate the internalization of *A. actinomycetemcomitans* into dendritic cells remains to be determined.

We found that the number of serotype b *A. actinomycetemcomitans* recovered from dendritic cells were at least five fold higher compared to the serotype a and serotype d strains (Figure 2.9). Different serotypes of *A. actinomycetemcomitans* are categorized by different composition of O-polysaccharide. The difference in the internalization among these strains may be due to the fact that VT1230 (serotype a) and VT1246 (serotype d) express different molecular forms of EmaA on the surface of the bacteria, as verified by immunoblotting and electron microscopy [8]. These molecules are suggested to vary in structure as compared with the serotype b or the full-length EmaA, due to the 279 amino acid deletion in the stalk domain in addition to the amino acid sequence difference in the adhesin moiety [9]. Therefore, structural differences may exist which impact the receptor-ligand interaction. In addition, the sugars associated with the EmaA structures of these serotypes may not be specific for the dendritic cell lectin receptors. Further investigations are warranted to address these questions.

Another possibility for the decreased recovery is that the serotypes of *A. actinomycetemcomitans* contain ~20% sequence variation in the genome, which may lead to potential phenotypical variations. The serotype b strain may express unique outer membrane proteins or virulence factors that are absent in serotype a or serotype d, which contributes to the internalization and intracellular survival of the serotype b strains.

In conclusion, we demonstrated by three independent yet complementary approaches that *A. actinomycetemcomitans* is internalized into dendritic cells and survive intracellularly. We also demonstrated that the surface glycoprotein EmaA is critical in

the internalization process, possibly by binding to the lectin receptors on dendritic cells through glycoprotein – lectin interaction and induce the subsequent uptake. In addition, bacterial internalization or survival is dependent on the serotype of the organism, possibly due to the different molecular forms or posttranslational modification of EmaA. The survival of *A. actinomycetemcomitans* in dendritic cells provides a putative mechanism for the immune evasion and dissemination of this oral bacterium from the periodontal tissues to distant organs of the body.

3.2. Future directions

Determine the role of EmaA molecular forms in dendritic cell internalization of *A. actinomycetemcomitans*. The genotype of *emaA* is closely associated with the serotype of the bacteria. Serotype b and c strains encode a full-length EmaA, whereas serotype a and d strains encode an intermediate form of EmaA [8]. Both forms of EmaA form surface structures and bind to collagen. However, the question still remains whether the intermediate form of EmaA mediates the internalization of *A. actinomycetemcomitans* into dendritic cells. We attempted to address this question in our research, however, the different survival rates of the different serotypes prevented us from dissecting the role of EmaA independent from the genome variation and different intracellular survival between different strains. In order to maintain a consistent genetic background among the strains in the study, we need to transform a plasmid harboring the gene encoding the intermediate form of EmaA into the *emaA* mutant serotype b strain. We will include the strain expressing the intermediate form of EmaA in the dendritic cell internalization assay and compare the internalization efficiency to the wild type and *emaA* mutant. We expect that the intermediate form of EmaA is able to mediate the

internalization of the bacterium into dendritic cells to a comparable level with the wild type strain.

Determine the role of EmaA glycosylation in dendritic cell internalization. EmaA is post-translationally glycosylated by the shared O-PS biosynthesis pathway [14]. The glycosylation of EmaA is required for the stability of the molecule and the collagen binding activity. We hypothesize that EmaA interacts with the lectin receptor(s) on dendritic cells via a glycoprotein-lectin interaction. To test our hypothesis, an *A. actinomycetemcomitans* strain expressing glycosylation deficient EmaA will be generated by disrupting the *waaL* gene. Overexpression of *emaA* in this mutant increases the level of EmaA to wild type levels but the defect in collagen binding still remains [15]. This suggests that the glycosylation is required for the function of EmaA. We will use this overexpression strain expressing the deglycosylated form of EmaA in the dendritic cell internalization assay to compare the dendritic cell internalization efficiency with the wild type strain and the *emaA* mutant strain. We expect the mutant harboring the deglycosylated EmaA will have a decreased rate of dendritic cell internalization that is comparable to the *emaA* mutant strain.

Determine the relationship between collagen binding and dendritic cell internalization of EmaA. The mature EmaA consists of three domains, including a N-terminal functional domain, which forms an ellipsoidal structure as is revealed by electron microscopy [9, 23]. The functional domain is followed by a long stalk domain, which contains sequences for the formation of coiled-coil structures. The C-terminus of the protein contains β-barrel folds that forms the membrane-anchor domain that facilitates the secretion of the protein out of the outer membrane. The collagen binding

activity of EmaA has been linked to the head domain of EmaA, which contains amino acids 70-384. Transforming plasmids harboring *emaA* sequences containing partial deletions were not able to complement the phenotype. There are multiple collagen binding motifs located in the head domain, which begin with either a serine or a glycine, three hydrophobic amino acids and ended with a glycine. A single point mutation in the collagen binding motif is sufficient to abolish the collagen binding activity of EmaA. However, we do not know whether the active domain for dendritic cell internalization overlaps with the collagen binding domain. Those isogenic mutants expressing the mutant EmaA will be used in the dendritic cell internalization assay and compared with the wild type and *emaA* mutant to determine the link between the collagen binding and dendritic cell internalization.

Determine the role of additional putative surface molecules in dendritic cell internalization. Besides EmaA, *A. actinomycetemcomitans* also express multiple surface proteins including the bundled fimbriae and non-fimbrial adhesins ApiA and Aae. Interestingly, the subunit of the fimbriae, Flp1, is also suggested to be glycosylated, possibly by a protein RcpC which is encoded downstream in the tad locus [19, 20]. Therefore, the fimbria of *A. actinomycetemcomitans* is another putative surface structure involved in dendritic cell internalization. The role of the fimbriae in dendritic cell internalization can be determined by comparing a *flp-1* mutant strain with the wild type in the dendritic cell internalization assay. An *rcpC* mutant will also be constructed to determine the role of Flp1 modification in the internalization process. The other two non-fimbrial adhesins, i.e. ApiA and Aae, are involved in the adhesion of epithelial cells [26].

The role of these two adhesins in dendritic cell internalization can be determined using the respective isogenic mutant in the same manner.

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